PATENT COOPERATION TREA . Y

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 26 February 2001 (26.02.01)	
International application No. PCT/GB00/02215	**Applicant's or agent's file reference** WN/NV/WCM.73
International filing date (day/month/year) 19 June 2000 (19.06.00)	**Priority date** (day/month/year) 17 June 1999 (17.06.99)
Applicant JONES, Derek, Leigh	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

12 January 2001 (12.01.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer S. Mafla
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 December 2000 (28.12.2000)

PCT

(10) International Publication Number
WO 00/78927 A3

(51) International Patent Classification[7]: C12N 5/06, C07K 14/765

(21) International Application Number: PCT/GB00/02215

(22) International Filing Date: 19 June 2000 (19.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9913979.2 17 June 1999 (17.06.1999) GB

(71) Applicant *(for all designated States except US)*: **UNIVERSITY OF WALES COLLEGE OF MEDICINE** [GB/GB]; Heath Park, Cardiff CF4 4XN (GB).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **JONES, Derek, Leigh** [GB/GB]; 3 Clos Derwen, Parkside Gardens, Roath, Cardiff CF23 5HJ (GB).

(74) Agents: **NEWELL, William, Joseph** et al.; Wynne-Jones, Laine and James, Morgan Arcade Chambers, 33 St. Mary Street, Cardiff CF10 1AF (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

(88) Date of publication of the international search report:
28 June 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/78927 A3

(54) Title: SPHEROID PREPARATION

(57) Abstract: A mixture or substance having a spheroid-forming activity is obtained by heat treating fetal calf serum at a temperature and for a period sufficient to impart spheroid-forming activity. Introduced into cell culture, the substance or mixture so obtained causes cells to grow in three-dimensional cultures as opposed to mono-layer. Also disclosed are kits for the production of said mixture or substance and further uses of said mixture or substance.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/02215

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/06 C07K14/765

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, EPO-Internal, PAJ, BIOSIS, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	N. MURAKAMI ET AL.: "Formation of ovary-like multitissue spheroids composed of isolated rat follicles in vitro." JOURNAL OF REPRODUCTION AND DEVELOPMENT, vol. 39, no. 4, 1993, pages 293-299, XP000971592 page 296, left-hand column, line 25 - line 31; tables 3,4 page 298, right-hand column, line 17 - line 24	24,26,34
A	US 4 237 033 A (E.M. SCATTERGOOD) 2 December 1980 (1980-12-02) claims	1-34

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 December 2000	08/01/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Ryckebosch, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int[redacted]nal Application No
P[redacted]GB 00/02215

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 529 659 A (SEIKAGAKU CORPORATION) 3 March 1991 (1991-03-03) cited in the application claims	1-34

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 00/02215

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4237033 A	02-12-1980	NONE	
EP 529659 A	03-03-1993	JP 5236951 A	17-09-1993
		AT 173012 T	15-11-1998
		AU 654376 B	03-11-1994
		AU 2138592 A	11-03-1993
		CA 2077175 A	01-03-1993
		DE 69227493 D	10-12-1998
		DE 69227493 T	06-05-1999
		FI 923874 A	01-03-1993
		KR 191363 B	15-06-1999
		NO 923376 A	01-03-1993
		US 5624839 A	29-04-1997

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 December 2000 (28.12.2000)

PCT

(10) International Publication Number
WO 00/78927 A2

(51) International Patent Classification[7]: C12N 5/00

(21) International Application Number: PCT/GB00/02215

(22) International Filing Date: 19 June 2000 (19.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9913979.2 17 June 1999 (17.06.1999) GB

(71) Applicant *(for all designated States except US)*: **UNIVERSITY OF WALES COLLEGE OF MEDICINE** [GB/GB]; Heath Park, Cardiff CF4 4XN (GB).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **JONES, Derek, Leigh** [GB/GB]; 3 Clos Derwen, Parkside Gardens, Roath, Cardiff CF23 5HJ (GB).

(74) Agents: **NEWELL, William, Joseph** et al.; Wynne-Jones, Laine and James, Morgan Arcade Chambers, 33 St. Mary Street, Cardiff CF10 1AF (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/78927 A2

(54) Title: SPHEROID PREPARATION

(57) Abstract: A mixture or substance having a spheroid-forming activity is obtained by heat treating fetal calf serum at a temperature and for a period sufficient to impart spheroid-forming activity. Introduced into cell culture, the substance or mixture so obtained causes cells to grow in three-dimensional cultures as opposed to mono-layer. Also disclosed are kits for the production of said mixture or substance and further uses of said mixture or substance.

Spheroid Preparation

This invention relates to a method of producing a substance or mixture having spheroid-forming activity from fetal calf serum and to methods of spheroid formation.

Spheroids are three-dimensional cultures of cells which are normally grown in suspension. A number of processes for formation of spheroids have been proposed, for example in US 5624839, but these have been found to be relatively complex. Although the term "spheroid" is often used conventionally to describe an object of approximately spherical shape, the term is used more broadly herein to describe any three-dimensional cell structure in which the cells are grown in suspension as opposed to in a mono-layer on a substrate. Thus the term spheroid embraces not only approximately spherical clusters of cells, but also string-like structures or lattice or net-like structures in which the cells form a three dimensional structure not of mono-layer form.

Spheroids in general are used in tissue culture research, for example.

According to the first aspect of the present invention, there is provided a method of producing a substance or mixture for use in spheroid formation, the method comprising heat treatment of fetal calf serum for a time and at a temperature sufficient to impart spheroid-forming activity to the resultant substance or mixture.

The heat treatment is preferably performed at a temperature between 60°C and 80°C, even more preferably

between 65°C and 75°C. However, it is envisaged that temperatures outside these ranges could be used, particularly below these ranges, although in such a case the incubation time would be longer. The heat treatment, for example, may be performed for between 30 minutes and 12 hours. It has been found that, for many batches of FCS, the optimum conditions for producing the substance or mixture are 70°C for 5 hours. However, different amounts of the substance or mixture may be produced at different temperatures and incubation times, with generally more being produced at the higher temperature and longer incubation time. Nevertheless, higher temperatures may give rise to too much coagulation of proteins in the serum, thus resulting in a loss of activity in the substance or mixture.

The method may further comprise the step of storing the resultant substance or mixture in aliquots at about -20°C.

According to a second aspect of the present invention, there is provided a substance or mixture for use in spheroid preparation formed by the method described above.

According to a further aspect of the present invention, there is provided a method of forming a spheroid comprising contacting in a vessel a cell culture with a substance or mixture formed by the method described above.

One or more cell types may be used, thus enabling the method to be used in the formation of heterospheroids in addition to homospheroids. Indeed, heterospheroids may be easily formed by adding several cell types in the required ratio.

The method of spheroid formation typically requires an overnight incubation period.

Spheroid size can be regulated by initial cell number, time of incubation and shape of culture vessel. Generally, small and medium sized spheroids (up to 100 micrometres), are formed after 24 hours and their size is increased thereafter mainly by fusion of spheroids rather than by cell growth.

The substance or mixture for use in spheroid preparation may, in one embodiment be coated on to the vessel, which may be formed of plastic. Alternatively, spheroid preparation may be carried out on uncoated vessels and, in such a case, a 5 to 10% solution of the substance or mixture for use in spheroid preparation may be added to a medium of the cell culture.

According to a further aspect of the present invention, there is provided an elongate spheroid comprising a plurality of cells arranged linearly.

The elongate spheroids are known as "string spheroids". Typically, the elongate spheroid may have a length of at least about 1cm, or preferably about 2cm. Typically, it has been found that elongate spheroids may be of the order of 0.2-0.5mm in diameter and may typically be 25cm long, containing 100,000 - 150,000 cells per cm length. However, it should be noted that elongate spheroids may be of 100cm in length or even more.

Again, the cells may be of one or more types, thus producing two homo- or hetero-string spheroids. In one

example, MCF7 and breast fibroblast cell lines have been prepared. One or more layers may be arranged around an inner elongate arrangement of cells. ECV cells have additionally been used to provide three cell layers in a triple string spheroid.

According to a further aspect of the present invention, there is provided a method of forming an elongate spheroid comprising forming a suspension by contacting a cell culture with a spheroid-forming substance or mixture formed by the method described above at the required concentration, placing the suspension in a tubular member, incubating the contents of the tubular member, and removing the elongate spheroid. Typically, the required concentration is in the range of 6 to 10 million cells per millilitre. In one embodiment, the tubular member may have an internal diameter of about 1mm. Typically, the tubular member may be in the form of a "butterfly" having a length of about 25cm and an outer diameter of 2mm, but any appropriate tubing, for example one of plastic and of suitable dimensions, could be used.

The method may further comprise the step of stretching the tubular member prior to incubation, and preferably holding the tube in a horizontal position.

According to a further aspect of the present invention, there is provided a kit for forming elongate spheroids, comprising a substance or mixture for use in spheroid formation formed by the method described above, and a culture vessel. The culture vessel may be tubular or of one

or more elongate components side by side or in a grid or lattice formation and having a v-sectioned base.

The kit may further comprise the cells which it is desired to form into an elongate spheroid, means for placing a suspension into the tubular member, means for removing the elongate spheroid from the tubular member and/or a stand for arranging the tubular member horizontally during incubation.

Many uses for the substance or mixture for use in spheroid formation according to this invention can be envisaged and examples include the following:

(i) It could be easily prepared as a commercially available product, either in its crude form or a purified form, for the production of homo- or heterospheroids in tissue culture research.

(ii) It could be used for the preparation of string spheroids made of different cell types such as fibroblasts, smooth muscle cells, and endothelial cells to make in-vitro veins.

(iii) It could be used for the preparation of keratinocyte/fibroblast and other skin cell mini-spheroids that could be attached to an artificial support for use as a sort of skin grafting. This could produce micro-islands of skin cells on the surface of open large area wounds. The closeness of the spheroids could be controlled to give optimum outgrowth and link up of skin islands, whilst initially allowing wound exuate etc. to pass between the islands.

(iv) It could form the basis for another angle on anti-

cancer therapy. When tumour cells are cultured as spheroids with the substance or mixture of the invention, their growth is slowed right down, and the cells stick together much more strongly (hence spheroid formation). It could therefore form the basis for an anti-metastatic factor and/or an agent to slow down or even stop tumour cell growth.

Thus, according to a further aspect of the present invention, there is provided the use of a substance or mixture for use in spheroid formation formed by the method described above in anti-cancer therapy.

The invention also extends to a polymer material comprising a polymer of one or more proteins contained in fetal calf serum, having a molecular weight of at least 2MDa and a spheroid forming activity.

In another aspect this invention provides a polymeric protein comprising a polymer of one or more proteins contained in fetal calf serum, having a molecular weight in excess of 2MDa and having spheroid forming activity.

In another aspect this invention provides a polymeric protein obtainable by heat treatment of fetal calf serum, whereby said polymeric protein is capable of spheroid forming activity.

In another aspect this invention provides the use of a polymeric protein for the preparation of skin cells selected from the group comprising keratinocytes and fibroblasts, for use in wound healing and/or skin grafting.

In another aspect this invention provides a method of elongate spheroid formation, which comprises providing an

elongate culture vessel having a generally V-shaped lower cross-section, introducing into said culture vessel a cell culture and a spheroid-forming substance or mixture, incubating the contents of said vessel and removing the elongate spheroid.

For convenience, in the description below, "Spefadel" is the name given to the spheroid forming substance or mixture of the present invention produced by heat treatment of commercially available fetal calf serum (FCS).

Although the invention has been defined above, it is to be understood that it includes any inventive combination of the features set out above or in the following description.

The invention may be performed in various ways, and specific examples will now be described, by way of example, with reference to the accompanying drawings, in which:

Figure 1 is a diagrammatic view, partially in cross section, of a triple string spheroid formed in accordance with the invention;

Figure 2 is a schematic perspective view of a culture vessel comprising a plurality of v-channels for string spheroid preparation;

Figure 3 is a cross-section of one of the v-channels showing the sedimented cells;

Figure 4 is a schematic top plan view of a culture vessel comprising a grid of v-channels, for spheroid preparation, and

Figure 5 is a schematic view of a grid string spheroid

produced in the vessel of Figure 4.

Example 1 - Preparation of "Spefadel"

Heat treatment of Fetal Calf Serum (FCS) in a waterbath at a temperature of between 65 and 75°C for 30mins to 7 hours gives rise to the substance or mixture known as 'Spefadel'. Different amounts of 'Spefadel' are produced at different temperatures and incubation times, with more 'Spefadel' being produced at the higher temperature and longer incubation time. The optimum conditions for the production of 'Spefadel' are 70°C for 5 hours. Higher temperatures, that is 75°C or above, give rise to too much coagulation of proteins in the serum, resulting in loss of 'Spefadel' activity.

No spheroid forming activity was found in FCS heat treated at 60°C for up to 4 hours, but there was 'Spefadel' activity after 7 hours incubation at this temperature.

It should be noted that the production and amount of 'Spefadel' may vary according to different batches of FCS. In this instance the temperature and length of treatment may be adjusted and the spheroid forming activity of the 'Spefadel' tested.

'Spefadel' is typically prepared by heating FCS at 70°C for 5 hours and storing in aliquots at -20°C until required.

Example 2 - Spheroid Preparation with Spefadel

Spheroids can be prepared from different cell lines in ordinary sterile tissue culture flasks/petri dishes or sterile non-tissue culture flasks/petri dishes. Spheroids can be prepared in flasks/dishes that have been pre-coated with 'Spefadel' for 24hrs or longer and then washed to remove any proteins etc. that have not adsorbed to the surface. Spheroids can be prepared in flasks/dishes in the presence of 1% to 10% 'Spefadel' in any standard tissue culture medium e.g. RPMI1640, DMEM, DMEM/F12 etc. Spheroids are only formed by cells in suspension and not by cells already attached to plastic tissue culture vessels. If 10% 'Spefadel' medium is added to subconfluent monolayers of all cell types tested, they continue to grow as monolayers and grow at almost the same rate as cells cultured with FCS supplemented medium. 'Spefadel' under these conditions is completely non-toxic to the cells.

Spheroid Preparation on Coated Plastic Vessels

The vessel to be used for the preparation of spheroids can be of virtually any type of non-toxic plastic suitable for cell culture, but must be sterile. Typical vessels used successfully have included Nunc/Sterilin 25cm^2 tissue culture flasks, Sterilin 90mm bacteriological plates, Falcon 25mm and 50mm tissue culture plates, and 96, 24 and 6 well Nunc microtest plates.

'Spefadel' at about 1ml/15sq cm of plastic surface was added and spread evenly over its surface. The vessel was then placed in a 37°C incubator for between 24 and 72 hours.

After the required time the 'Spefadel' was removed and the surface of the vessel was given 3x 10min washes with 5ml aliquots of serum free medium (such as DMEM/F12) before adding about 4ml of the same medium containing 1mg/ml Bovine Serum Albumin (BSA), penicillin (100units/ml), streptomycin (100μg/ml) and fungizone (2μg/ml) (these three antibiotics together at these concentrations are known as PSF).

Breast tumour cell lines such as MCF7, MDA231 and BT474, human fibroblasts from breast and skin and a bladder cancer cell line such as ECV all available from ECACC or ATCC have all been used to prepare spheroids on coated plastic vessels. Basically cells were cultured as monolayers in a standard fashion in 25cm^2 Nunc tissue culture flasks with DMEM/F12 containing 10% FCS and PSF in a 37°C incubator with 5% CO_2, until almost confluent when they were made into a cell suspension with trypsin/EDTA (0.05% porcine trypsin and 0.05% EDTA in phosphate buffered saline). Cells were made up in complete 10% FCS medium and counted before centrifugation at 400G and resuspension at 1 million cells/ml in SFM with PSF and BSA. For homospheroids about 1ml of the cell suspension was added to each 25cm^2 flask and left in the CO_2 incubator for 24 hrs, after which time spheroids were formed as clusters of 20 to hundreds or even thousands of cells. Initially small spheroids were formed by attachment of cells to each other and then larger spheroids were formed by the fusion of small spheroids. Generally speaking spheroid size can be modulated by the number of cells used and the length of time they are left together. Increasing either incubation time or cell number

usually gives an increase in the size of spheroids.

Heterospheroids with different ratios of cells can easily be prepared. For example the addition of 250 000 fibroblast cells to 1 million MCF7 cells gives rise to spheroids with 4 times as many MCF7 cells as fibroblasts. The fibroblasts always end up at the centre of the heterospheroid surrounded by MCF7 cells, regardless of cell number ratios or even if the fibroblasts are added to MCF7 cells that have already formed spheroids.

Spheroid Preparation on Uncoated Plastic Vessels

The culture vessels and basic medium to be used for the preparation of spheroids on uncoated plastic are exactly the same as those used for the coated method. The main differences in the method is the addition of 5 to 10% 'Spefadel' to the basic culture medium instead of 1mg/ml BSA. All other conditions used for the preparation of spheroids on coated plastic apply to the preparation of spheroids on uncoated plastic.

Example 3 - String Spheroid Preparation

String spheroids are made from cells prepared in suspension in 10% 'Spefadel', similar to those for spheroids on uncoated plastic. In order for cells to form a complete string they have to be seeded at a certain concentration so that there are enough cells present to form a complete string but not too many cells present so as to use up all the nutrients and give rise to excessive cell death.

Actual cell numbers used for string spheroids also

depend on the cell type used and some cells such as fibroblasts only form short lengths of string spheroid, probably due to weaker connections between the cells, when compared to cells of epithelial type such as MCF7 or BT474 tumour cell lines.

Cells are prepared in suspension in 10% 'Spefadel' medium as previously described. For most cell types the optimum cell number for string spheroid preparation is between 6 and 10 million cells/ml. For MCF7 and BT474 cells the optimum is about 8 million cells/ml. Once the cells are prepared in suspension at the required concentration they are ready to be placed in a disposable sterile string spheroid apparatus. The apparatus currently used is very simple and consists of a sterile 21 gauge "butterfly" (Registered Trade Mark) with a tube length of about 25cms of internal/external diameter about 1mm and 2mm respectively. The "butterfly" is a hollow needle connected to a luer syringe connector by a hollow plastic tube. Other sizes may be used.

The method for string spheroid preparation of MCF7 cells will now be described.

Prepare a suspension of 8 million MCF7 cells/ml in 10% 'Spefadel' as already described. Take a 1ml disposable syringe and suck up 0.65ml of 10% 'Spefadel' medium and then, taking care not to get any air bubbles, suck up slowly 0.35ml of the MCF7 cell suspension, whilst holding the syringe vertical, so that it forms a separate layer in the syringe. Connect the syringe to the butterfly and slowly press the syringe whilst still holding vertical until the

suspension reaches the end of the plastic butterfly tube (care must be taken to avoid the introduction of air bubbles, as these will cause breaks in the string spheroid) which will be about 0.35ml in volume. Immediately slightly stretch the tubing over a horizontal holding frame so that the tube is held in a straight line in a horizontal position. Several string spheroids are usually made at any time and the current holding frame can accommodate up to 6 tubes. The whole process is done aseptically in a laminar flow hood to minimise contamination by microorganisms. The frame and tubes are now placed in a 5% CO_2 incubator and left overnight (18 hours). After this time the tubes are removed singly and cleaned with a steriswab before cutting the plastic tube aseptically close to the needle end of the butterfly. The tube contents are then ejected slowly (by gently pressing the syringe to push the remaining 0.7ml of medium through the tube) into 10ml of 1% 'Spefadel' medium in a 90mm sterile plastic plate. The result is a 'string spheroid' of MCF7 cells about 20cm long containing about 150,000 cells per cm length.

Hetero-string spheroids containing 2 and 3 cell types have also been prepared using the same method. Heterospheroids containing MCF7 and breast fibroblast cell lines have been prepared using cell suspensions containing 6 million MCF7 and 3 million fibroblasts per ml of medium. In this hetero-string spheroid the fibroblasts are always at the centre surrounded by MCF7 cells. In the triple string spheroid ECV cells were also present in the cell suspension and these formed a layer of cells around the MCF7 cells to

give three cell layers as shown in Figure 1.

Referring now to Figures 2 and 3, an alternative method of preparation of string spheroids will now be described.

Example 4

In this method string spheroids are prepared using cells suspended in 10% Spefadel in DMEM/F12 medium. It requires the use of a special shaped culture vessel 20 made out of polystyrene, polycarbonate or any material compatible with cell culture and which has a v-shaped corrugated profile as seen in Figures 2 and 3. The dimensions for the 'V' profile used may vary from between 5mm and 15mm wide (w) and 5mm and 15mm tall (h). The length of the 'V' channel used may typically be up to 15cm but longer lengths can be used. Just one V-profile may be used, but usually several 'V' profiles are joined to each other giving rise to a vessel containing a series of parallel 'V' channels. The ends of the 'V' channel are blocked off by walls 22.

In this method a cell suspension in 10% Spefadel DMEM/F12 medium is placed in the 'V' channel which is then placed on a level surface in an incubator. The cells fall through the liquid due to gravity, and because the sides of the channel are sloped nearly all the cells fall to the bottom groove of the channel to give a continuous length of sedimented cells as seen in Figure 3.

After 24 to 36 hours the cells attach to each other to give a string spheroid which can be gently removed from the 'V' channel or left in situ where the medium can be carefully changed when required. Homo and hetero-string spheroids

can be prepared by this method using one cell type or mixed cell type cell suspension.

Cell concentrations used for this method vary depending on the cell type and the volume of liquid placed in the 'V' channel. Generally cell concentrations are adjusted so that when the cells are sedimented there are about 100000 to 200000 cells for each centimetre in length of the channel. Thus a 'V' channel of 15cm length containing 5ml of medium might typically require a cell concentration of 0.3 to 0.6 million cells/ml.

Example 5

This is another variation of the 'V' channel method. Referring now to Figures 4 and 5, the main modification is the 'V' channel vessel 30 in which the cells are sedimented. It consists of two sets of 'V' channels (32, 34) at right angles to each other so that a grid of interconnected 'V' channels is formed. Dimension of the 'V' channel cross section, cell suspensions and incubation conditions used may be the same as those for the linear 'V' channel method of Example 4. When cells are placed in the 'V' channel grid they sediment and form string spheroids at right angles to each other which are joined where their paths cross. This results in the formation of a 'Grid string spheroid' (Figure 5), the dimensions of which depend on the spacing between the 'V' channels in the vessel.

A possible advantage of the 'V' channel methods of Examples 4 and 5 over the tube method for string spheroid

preparation is that after string spheroid formation by a first cell type one could remove the medium and add a cell suspension of a second cell type. This would allow the sequential addition of different cell types giving rise to another method for hetero-string spheroid preparation.

List of Abbreviations

abbreviation	description
BSA	Bovine serum albumin
BT474	Breast tumour cell line
CO2	Carbon dioxide
DMEM	Dulbeccos Minimal Essential Medium
DMEM/F12	Dulbeccos Minimal Essential Medium/Hams F12 tissue culture medium
ECV	Bladder Cancer cell line
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal Calf Serum
MCF7	Breast tumour cell line
PFS	penicillin (100u/ml), fungizone (2μg/ml) and streptomycin (100μg/ml)
RPMI1640	Roswell Park Memorial Institute 1640 tissue culture medium

Claims

1. A method of producing a substance or mixture for use in spheroid formation, the method comprising heat treatment of Fetal Calf Serum for a time and at a temperature sufficient to impart spheroid-forming activity to the resultant substance or mixture.

2. A method according to Claim 1, wherein the heat treatment is performed at a temperature between 60°C and 80°C.

3. A method according to Claim 1 or 2, wherein the heat treatment is performed at a temperature between 65°C and 75°C.

4. A method according to any preceding claim, wherein the heat treatment is performed for between 30 minutes and 12 hours.

5. A method according to any preceding claim, wherein the heat treatment is performed at a temperature of 70°C for about five hours.

6. A method according to any preceding claim, further comprising the step of storing the resultant substance or mixture in aliquots at about -20°C.

7. A substance or mixture for use in spheroid preparation formed by the method according to any preceding claim.

8. A method of spheroid formation comprising contacting in a vessel a cell culture with a substance or mixture formed by the method of any one of claims 1 to 6.

9. A method according to Claim 8, wherein the spheroid-forming substance or mixture is coated on the vessel.

10. A method according to Claim 8, wherein a 5 to 10%

solution of the spheroid-forming substance or mixture is added to a medium of the cell culture.

11. A method according to any of Claims 8 to 10, wherein the cell culture comprises more than one cell type, whereby a hetero-spheroid is formed.

12. An elongate spheroid comprising a plurality of cells arranged linearly.

13. An elongate spheroid according to Claim 12 which has a length of at least 1cm.

14. An elongate spheroid according to Claim 12 or 13, which contains 100,000- 200,000 cells per cm length.

15. An elongate spheroid according to any of Claims 12 to 14, comprising more than one cell type.

16. An elongate hetero-spheroid according to any of Claims 12 to 15, comprising an elongate core of cells of one type with one or more layers of cells of a different type arranged around said core.

17. An elongate hetero-spheroid comprising MCF7 and breast fibroblast cells.

18. A method of forming an elongate spheroid comprising form a suspension by contacting a cell culture with a spheroid-forming substance or mixture at the required concentration, placing the suspension in a tubular member, incubating the contents of the tubular member, and removing the elongate spheroid.

19. A method according to Claim 18, wherein the required concentration is in the range of 6 to 10 million cells/ml.

20. A method according to Claim 18 or 19, wherein the tubular member has an internal diameter of about 1mm.

21. A method according to any one of claims 18 to 20, further comprising the step of stretching the tubular member prior to the incubation.

22. A kit for forming elongate spheroids comprising a spheroid forming substance or mixture and a tubular member.

23. The use of a spheroid-forming substance or mixture formed by the method of any one of claims 1 to 11 in anti-cancer therapy.

24. A polymeric protein comprising a polymer of one or more proteins contained in fetal calf serum, having a molecular weight in excess of 2MDa and having spheroid forming activity.

25. A polymeric protein obtainable by heat treatment of fetal calf serum, whereby said polymeric protein is capable of spheroid forming activity.

26. The use of a polymeric protein according to Claim 24 or 25 for the production of spheroids for tissue culture.

27. The use of a polymeric protein according to Claims 24 or 25 for the production of spheroids made up of one or more of fibroblasts, smooth muscle cells and bladder cancer cells.

28. The use of a polymeric protein according to Claim 24 or Claim 25 for the preparation of skin cells selected from the group comprising keratinocytes and fibroblasts, for use in wound healing and/or skin grafting.

29. A method of elongate spheroid formation, which comprises providing an elongate culture vessel having a generally V-shaped lower cross-section, introducing into said culture vessel a cell culture and a spheroid-forming

substance or mixture, incubating the contents of said vessel and removing the elongate spheroid.

30. A method of producing a spheroid making up a grid structure, which comprises providing a corresponding culture vessel defining a grid in which the grid elements are of V-section, and introducing into said culture vessel a cell culture and a spheroid-forming substance or mixture, incubating the contents of said vessel and removing a spheroid of grid-like structure.

31. A method according to Claim 29 or 30, wherein said incubation is for a period of 24 to 36 hours.

32. A method according to Claim 29 or 30, wherein said V-shaped section defines an inclined angle in the range of from 20° to 120°.

33. A kit for forming elongate spheroids or a grid-like structure thereof, omprising a culture vessal having an elongate portion with a generally V-shaped lower cross-section, and a spheroid-forming substance or mixture.

34. A method of spheroid formation comprising contacting in a vessel one or more cell cultures with a polymeric protein according to Claim 24 or 25.

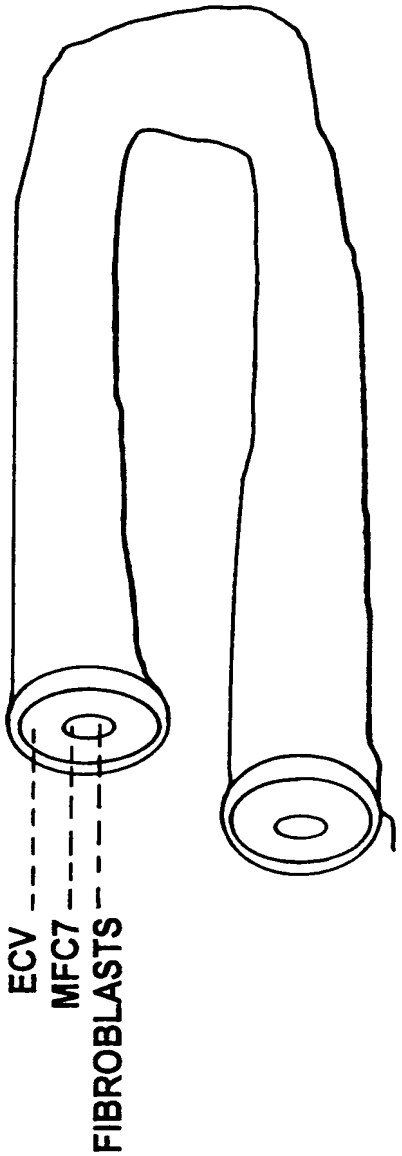


Fig. 1

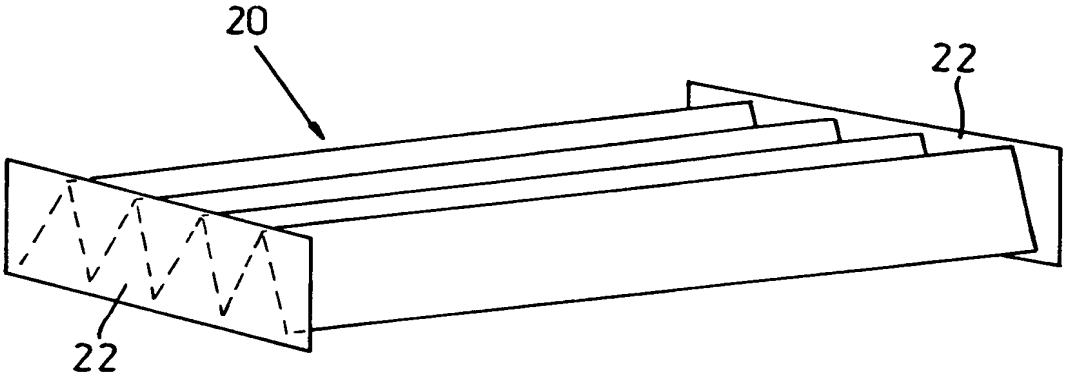


Fig. 2

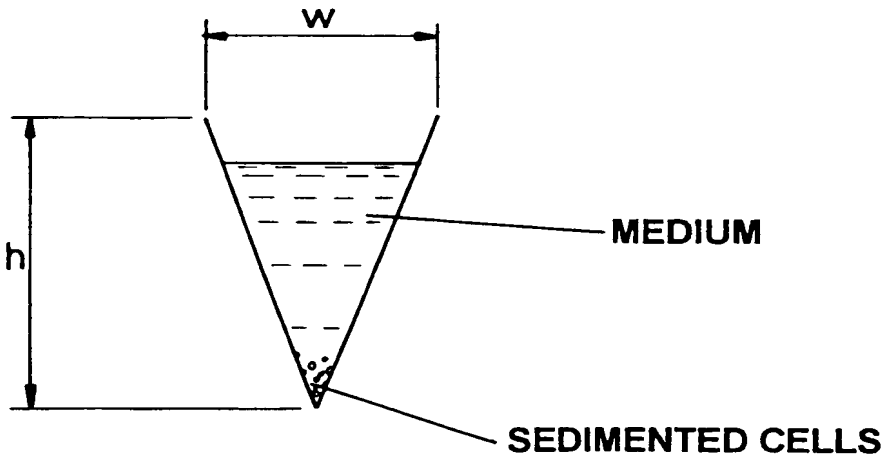


Fig. 3

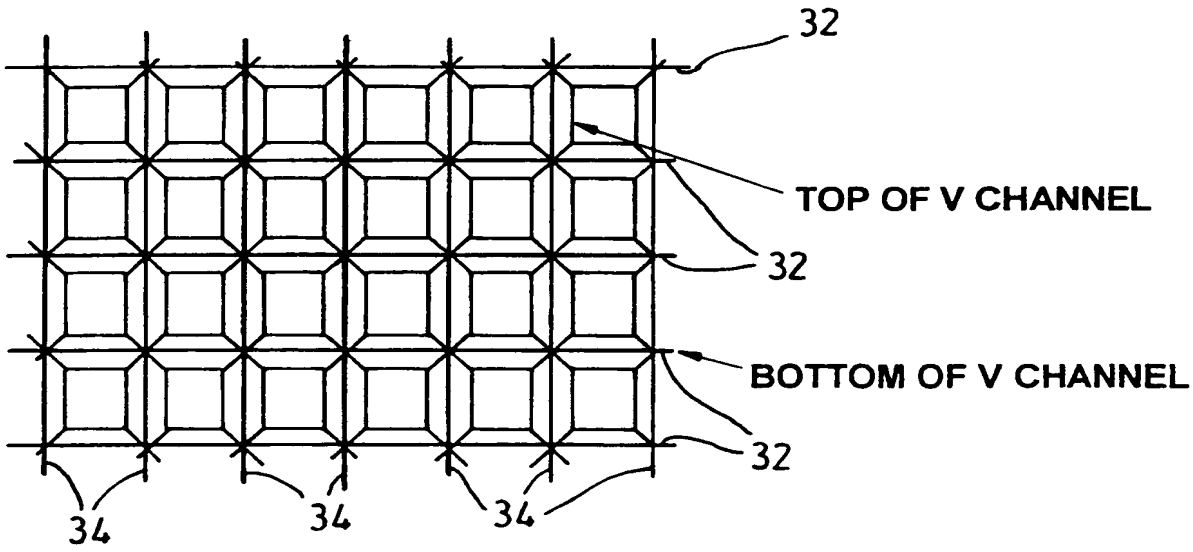
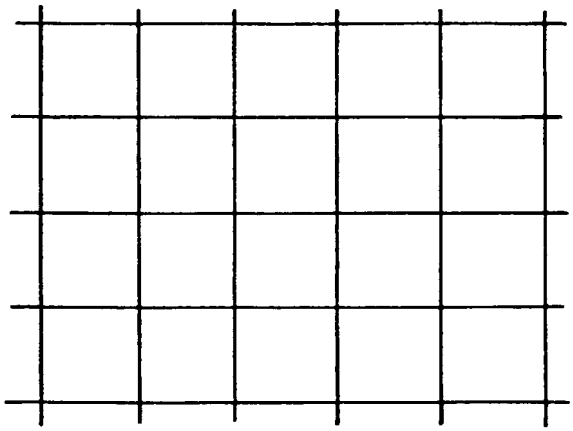


Fig. 4



PARALLEL JOINED STRING SPHEROIDS IN THE FORM OF A GRID (FORMED IN A GRID 'V' CHANNEL VESSEL WHERE THE BOTTOM OF THE 'V' CHANNELS ARE SPACED 1cm APART)

Fig. 5

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/02215

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/06 C07K14/765

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, EPO-Internal, PAJ, BIOSIS, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	N. MURAKAMI ET AL.: "Formation of ovary-like multitissue spheroids composed of isolated rat follicles in vitro." JOURNAL OF REPRODUCTION AND DEVELOPMENT, vol. 39, no. 4, 1993, pages 293-299, XP000971592 page 296, left-hand column, line 25 - line 31; tables 3,4 page 298, right-hand column, line 17 - line 24 ---	24,26,34
A	US 4 237 033 A (E.M. SCATTERGOOD) 2 December 1980 (1980-12-02) claims --- -/--	1-34

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 December 2000	08/01/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ryckebosch, A

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 529 659 A (SEIKAGAKU CORPORATION) 3 March 1991 (1991-03-03) cited in the application claims	1-34

INTERNATIONAL SEARCH REPORT

Information on patent family members

nal Application No
PCT/GB 00/02215

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4237033 A	02-12-1980	NONE	
EP 529659 A	03-03-1993	JP 5236951 A	17-09-1993
		AT 173012 T	15-11-1998
		AU 654376 B	03-11-1994
		AU 2138592 A	11-03-1993
		CA 2077175 A	01-03-1993
		DE 69227493 D	10-12-1998
		DE 69227493 T	06-05-1999
		FI 923874 A	01-03-1993
		KR 191363 B	15-06-1999
		NO 923376 A	01-03-1993
		US 5624839 A	29-04-1997

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
--- ---		

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/02215	19/06/2000	17/06/1999

International Patent Classification (IPC) or national classification and IPC
C12N5/00

Applicant
UNIVERSITY OF WALES COLLEGE OF MEDICINE et al.

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
12/01/2001	17.07.2001

Name and mailing address of the international preliminary examining authority:

European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer

Bretherick, J

Telephone No. +49 89 2399 8415

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/02215

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-17 as originally filed

Claims, No.:

1-34 as originally filed

Drawings, sheets:

1/5-5/5 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	2-6,9,12-34
	No:	Claims	1,7,8,10,11
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-34
Industrial applicability (IA)	Yes:	Claims	1-22, 24-27,29-34
	No:	Claims	23,28, opinion reserved

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

1. **Regarding Part VIII, Art. 5 and 6 PCT:**

a. The terms " spheroid formation" and "spheroid-forming" activity as used in the claimed subject matter are not clear from an immediate reading of the claims. Also, the term "spheroid" has been used in a much wider context than that which is understood in the art (page 1 lines 5-8, compared to lines 9-17 of this current application specification). There is also no definition of the term "hetero-spheroid" (claim 11), nor of the term "tubular-member" (claims 18, et seq., 22).

b. Due to the above unclear definition, the polymeric proteins of claims 24 and 25, as well as their dependencies are not clear also, since these claims do not enable the identification of the proteins in terms of other parameters, such as structure.

The definition of these terms and others relating thereto should be clarified in the light of Art. 6 PCT.

c. There is inadequate information in the application in support of claim 23. Information pertaining to therapeutic activity is lacking, as is a detailed therapeutic method protocol. The claim is considered to be purely speculative and thus deficient under Art. 5 PCT.

2. **Regarding Part V, Art. 33 PCT:**

a. The following art has been cited in the International Search Report:

D1: N. MURAKAMI ET AL.: 'Formation of ovary-like multitissue spheroids composed of isolated rat follicles in vitro.' JOURNAL OF REPRODUCTION AND DEVELOPMENT, vol. 39, no. 4, 1993, pages 293-299, XP000971592
D2: US-A-4 237 033 (E.M. SCATTERGOOD) 2 December 1980 (1980-12-02)
D3: EP-A-0 529 659 (SEIKAGAKU CORPORATION) 3 March 1991 (1991-03-03) cited in the application

b. Claim 1 is not new (Art. 33(1)(2) PCT). Foetal calf serum is known to result in spheroid production in ovarian cell culture. The "heat treatment" in this case is

maintenance at room temperature. (D1, results, page 294 to top of page 295 page 296, LH column, 3rd. paragraph). This approach is valid, since a further definition of "heat treatment" is not given in the claim.

The same applies, **mutatis mutandis**, to claims 7, 8,10 and 11.

c. The remaining claimed subject-matter dependent upon claim 1 and its dependencies is not considered to comprise an inventive step in the light of the remaining cited art. Of particular relevance is D2, which shows that heat-treatment of FCS within the temperature range claimed causes the formation of protein polymers which enable more efficient colonisation of microcarrier beads by tissue culture cells. D3 shows that these conditions can also be obtained using other products to form hepatic spheroids.

d. Claims 23 and 28 are directed to therapeutic methods. An opinion as to the industrial applicability of these claims is reserved in view of the lack of unified, common guidelines within the PCT for the assessment of such claims.

PCT

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

To:
NEWELL, William, Joseph
Wynne-Jones, Laine and James
Morgan Arcade Chambers
33 St. Mary Street
Cardiff CF10 1AF
ROYAUME-UNI

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

Date of mailing (day/month/year) 28 December 2000 (28.12.00)	
Applicant's or agent's file reference WN/NV/WCM.73	**IMPORTANT NOTICE**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/GB00/02215	19 June 2000 (19.06.00)	17 June 1999 (17.06.99)

Applicant
UNIVERSITY OF WALES COLLEGE OF MEDICINE et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AG,AU,BZ,DZ,KP,KR,MZ,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CN,CR,CU,CZ,DE,DK,DM,EA,EE,EP,ES,FI,GB,GD,
   GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,
   NO,NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,ZW
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   28 December 2000 (28.12.00) under No. WO 00/78927

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra

Telephone No. (41-22) 338.83.38